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ANK repeats were first found in the Swi6 transcription factor of *Saccharomyces cerevisiae* and since then were identified in many proteins, including oncogenes and tumor suppressors We have previously described generation of random mutations within the ANK repeats of Swi6 that render the protein temperature sensitive in its ability to activate *HO* transcription. Two of these *SWI6* mutants were used in a screen for high copy suppressors of this phenotype. We found that *MSN1*, which encodes a transcriptional activator, and *NHP6A*, which encodes an HMG-like protein, are able to suppress defective Swi6 function. Both of these gene products are involved in *HO* transcription, and Nhp6A may also be involved in *CLN1* transcription. Moreover, since overexpression of *NHP6A* can suppress caffeine sensitivity of one of the *SWI6* ANK mutants, *swi6-405*, other *SWI6*-dependent genes may also be affected by Nhp6A. We hypothesize that Nhp6A and Msn1 modulate Swi6-dependent gene transcription indirectly, through effects on chromatin structure or other transcription factors, since we have not been able to demonstrate that either Msn1 or Nhp6A interact with the Swi4/Swi6 complex.

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Linda Breeden June 22, 1998

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The Role of the ankyrin repeats in the Swi4/Swi6 transcription complex of budding yeast.

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INTRODUCTION

Over one hundred proteins have been identified that contain ankyrin repeats, including several proteins in which the ankyrin repeat motifs may play a role in tumor development. These include Bcl-3, *int*-3, TAN-1, and p16. p16 (INK4) is composed of only a series of four ankyrin repeats and is one member of the cyclin dependent kinase inhibitor family of proteins. p16 can compete with cyclin D and displace the cyclin subunit from its kinase binding site, thereby inhibiting G1 to S progression through the cell cycle. Mutations in p16 have been noted in a variety of human tumors and tumor cell lines, in addition, mutations have been found in the germline of families predisposed to melanoma (1). Ankyrin repeats are presumed to have a role in protein-protein interaction, though these interactions are largely uncharacterized and highly diverse. Circular dichroism and NMR studies of the tumor suppressor protein $p16^{INK4A}$, which consists of four ankyrin repeats, have confirmed the predominantly alpha-helical nature of the ankyrin repeat region (2). The crystal structure of the p53 core domain bound to 53BP2 reveals that an ankyrin repeat consists of a B hairpin and 2 α helices (3).

The ankyrin/Swi6/Cdc10 repeat was first identified in a family of yeast transcription factors (4,5). Included within this family are the *Schizosaccharomyces pombe* transcription factors Cdc10, Res1, and Res2, and the *Saccharomyces cerevisiae* transcription factors Swi6, Swi4, and Mbp1 (Figure 1); members of these groups associate with one another through their C-termini (6,7). The target genes for these transcription factors are expressed at the G1/S transition in yeast, and include DNA synthesis genes (8,9), *HO* endonuclease, and the G1 cyclins (10,11) that are required for progression through the yeast mitotic cell cycle. Swi6 is one of the transcription factors that regulates gene expression during G1/S, either as a complex with Mbp1, with which it binds MCB (ACGCGTNA) DNA elements (8,9,12), or in combination with Swi4, with which it binds SCB (CACGAAAA) elements (13-15) as well as MCB-like elements (16). Swi6 is not known to bind DNA directly, rather, both Mbp1 and Swi4 confer the DNA binding ability to the complex through their N-termini.

A common central motif consisting of four and one half ankyrin repeats is present among the known G/S specific yeast transcription factors. Ankyrin repeats typically occur as four or more continuous copies of the ankyrin motif, which is a thirty-three amino acid sequence characterized by the consensus structure:

-------t-otLHhAh--tt-thht-LLt-t—

where "t" indicates a turn-like or polar residue, "h" a hydrophobic residue, "o" a serine or threonine residue, and capital letters indicate highly conserved amino acids (17). These repeats are not required for Swi4/Swi6 interaction, but are required for function. This may indicate a role for ankyrin repeats in recruiting additional proteins to the transcription complex. With this in mind, we have focused our efforts upon genetic strategies to identify ANK repeat-interacting proteins and to define their role, if any, in DNA binding and activation of transcription. The central goal of these studies has been to determine the function of ankyrin repeats in Swi6 and to then ask if this is a

common function of all ankyrin repeats. If there is a conserved function for these repeated motifs, then understanding what that is will provide important new insight into the many oncogenes and tumor suppressors that also contain these repeats.

One major paper has been published, which reports the results of some of this work (see accompanying reprint). This work was largely summarized in the last annual report. A second paper has been recently submitted to Genetics. This work will be summarized in this report and pertains specifically to aim 5, which is to identify second site suppressors, either within the Swi6 protein, or within associated proteins. Our purpose has been to identify proteins which interact with the ANK repeats of Swi6. Mutations have been identified within the ANK repeat, and these have been used to search for interacting gene products using suppressor analysis.

Experimental procedures

Growth conditions: All rich (YEPD) and minimal (YC) media and growth conditions were as described previously (18). Temperature sensitive ANK mutant strains were cultivated at 30° and shifted to 37° for 8-12 hours when grown in liquid media. When grown on plates, they were incubated at 37° for the whole period of growth.

DNA, RNA and protein analysis: FACS analysis of yeast cells was done as described in (19) and data were analysed using CellQuest software. Procedures for RNA isolation and S1 protection were performed as described previously (18). Protein extract preparation, immunoprecipitation and Western blotting were done as described before (6,20).

In vitro transcription and translation: The plasmid pBD972 was used for in vitro translation of Swi4 (21). pBD972 was added to a TNT Rabbit Reticulocyte lysate coupled transcription translation system (Promega) along with 20-50 ng of the recombinant Swi6 purified from *E. coli* (6). Reactions were carried out according to manufacturer's recommendations with cold amino acids. Reaction products were added directly to *HO* promoter DNA binding reactions or loaded onto SDS PAGE.

Gst fusion and purification from yeast or bacterial cells: To construct *GST* fusions, *MSN1* and *NHP6A* were generated by polymerase chain reaction (PCR) from pM-4 (pBD2050) and pN-5 (pBD2055) respectively, using M13 reverse primer and BL138 5'GGATCCATGGTCACCCCAAGAG3' primer for *NHP6A*, and M13 reverse and BL137 5'CCGGATCCATGGCAAGTAACC3' primers for *MSN1*.

To purify Gst fusions from E. coli, pBD2064 and pBD2062 were transformed into DH5α cells and the resulting strains were treated according to Pharmacia Biotech Gene Fusion System protocols. Bacterial cultures were grown to OD 0.6, and fusion protein expression was induced by 0.1mM IPTG for 2 hours. Cells were then harvested, sonicated, centrifuged, and extracts were incubated with glutathione Sepharose 4B beads (Sigma) for 30 min at 4°. To determine if the Gst fusions were capable of interacting with Swi6, these glutathione beads with fusion proteins immobilized on them were incubated with recombinant Swi6 or in vitro translated Swi4/Swi6 complex, washed, boiled and loaded onto SDS PAGE.

To obtain Gst fusions from yeast, fusion plasmids were transformed into W303-1a strain. The resulting strains were grown in selective media with raffinose overnight and then expression of the fusions was induced by galactose for 3-4 hours. Cells were harvested, protein extracts were prepared as described before (20) and incubated for 1 hour with glutathione beads in GST buffer containing protease inhibitors (100mM Tris HCl pH8.0, 100mM NaCl, 0.2% NP40 with 1mM PMSF, 1μg/ml leupeptin, 1μg/ml pepstatin A). Beads were then washed in three or four changes of GST buffer. To elute fusion proteins from the beads, the beads were resuspended in 50μl of glutathione buffer, prepared according to the Pharmacia Biotech protocol. Fusions were eluted for 15 min at room temperature.

Gel retardation: Gel retardation analysis was performed exactly as described (6,21). When the in vitro translated Swi4/Swi6 complex was bound to DNA, little (0.2 -0.5 μg) or no nonspecific competitor dI-dC was added. The binding pattern was the same, regardless of whether dI-dC was present in the reaction, or not. Thrombin or glutathione eluates of the Gst-Nhp6A fusion were directly added to DNA binding reactions with *HO* promoter fragment. No dI-dC competitor was used in these reactions since Nhp6A is a nonspecific DNA-binder (22) and can be competed from *HO* DNA by dI-dC (data not shown).

Results and Discussion

Screen for high copy suppressors of temperature sensitive *ho::LacZ* expression phenotype of *swi6-405* and *swi6-406* mutants: To better understand the function of the ANK repeats of Swi6 we carried out random mutagenesis of the ANK repeat-encoding region of the *SWI6* gene (21). Two of these mutants, *swi6-405* (N330T, N500Y) and *swi6-406* (T326I, T402S), were used in this study. Both mutants express Swi6 protein at the nonpermissive temperature, however, the level of Swi6 is reduced as compared to the wild type protein level. There is no *ho::lacZ* activity detected in *swi6Δ* strains expressing Swi6-405 or Swi6-406 from the CEN plasmid pRS316 at 37°, but at 30° and 25°, they confer partial activity as judged by cell morphology and *ho::lacZ* transcript levels (data not shown and see below). In band shift assays, Swi4/Swi6-405 complex is less active in binding to SCB elements than the wild type complex, and Swi4/Swi6-406 complex has an altered mobility (21).

The mutated *SWI6* genes were integrated at the *LEU2* locus of the *swi6Δ ho::lacZ* strain, giving rise to strains BY1954 (*swi6-405*) and BY1956 (*swi6-406*). These strains were transformed with a 2μm-based yeast genomic library (23) and about 60,000 transformants were obtained for each. Colonies were grown at 30°C for the first two days upon transformation and then incubated at 37°C overnight. Colonies were transferred to nitrocellulose filters and assayed for β–galactosidase activity using the X-gal filter assay (24). Transformants that developed blue color above the background were selected. Library plasmids were isolated out of these cells, retransformed into BY1954 or BY1956 strains and reassayed to confirm suppression.

We recovered a total of 8 suppressor plasmids from *swi6-406* and 19 from *swi6-405* transformants. These are listed in Table 1. The *SWI6* gene was isolated five times in

the screen with *swi6-406* and three times with *swi6-405*. Two suppressors (c2a and c6) were not pursued further because they activated *ho::lacZ* expression equally strongly in *swi6-405* and *swi6Δ* cells and thus were completely independent of Swi6. Most of the suppressors (c2, c4, c5, c9, c12, c19, c23) suppressed the *ho::lacZ* expression defect to some extent in the absence of Swi6, but only one (c19) could suppress in the absence of Swi4. This requirement for Swi4 suggests that the majority of suppressors enhance Swi4-mediated activation , rather than by causing a general derepression of transcription. c15 showed no suppression of the *ho::lacZ* transcription defect in *swi4* or *swi6* deletion strains and thus was the best candidate for an allele-specific suppressor.
Previously identified genes, *MSN1* and *NHP6A,* were responsible for suppression phenotype of c4 and c15, respectively. *MSN1* was originally cloned as a high copy suppressor of a temperature sensitive *SNF1* kinase mutant for its ability to restore *SUC2* expression (25). It acts as a transcriptional activator when fused to LexA and does not have any specific DNA binding activity (25). *NHP6A* has also been identified previously (26) and encodes an HMG1-like small protein, which binds DNA nonspecifically, and is capable of bending DNA (22).

Table 1.High copy suppressors of *SWI6* ankyrin repeat mutants.

A. Suppressors isolated from *swi6-406* strain

Plasmid	Suppressor	Suppression of *ho::lacZ* expression in strains with the following mutations		
		swi6-406	*swi6Δ*	*swi4Δ*
c1 (5)	*SWI6*	+	+	n/a
c2a (1)	n/d	+	+	n/a
c4 (1)	*MSN1*	+	+/-	-
c6 (1)	n/d	+	+	n/a

B. Suppressors isolated from *swi6-405* strain

		swi6-405	*swi6Δ*	*swi4Δ*
c1 (1), c14 (2)	*SWI6*	+	+	n/a
c2 (1)	n/d	+	+/-	-
c4 (8), c5 (1), c9 (1), c12 (1)	*MSN1*	+	+/-	-
c15 (1)	*NHP6A*	+	-	-
c19 (1)	n/d	+	+/-	+/-
c23 (1)	n/d	+	+/-	-

The strains indicated below were transformed with vectors or with vectors bearing suppressing genes, grown on plates at 37° and screened for β–galactosidase activity with X-gal assays. Numbers in brackets by the clone name indicate the number of times this clone was isolated. n/a - not applicable, n/d - not determined.

***MSN1* and *NHP6A* suppress the *HO* transcription defect of *SWI6* ANK mutants:** Since *NHP6A* (c15) was incapable of bypassing Swi6 function, we sought to determine if *NHP6A* suppression was specific to the *swi6-405* allele of *SWI6*. The *NHP6A* gene on pRS426 was transformed into strains with different mutant alleles of *SWI6* and X-gal filter assays were performed. All these mutants are expressed at nonpermissive temperature (37°) (21) and data not shown). *swi6-Δ21* encodes a nonconditional and highly defective Swi6 protein (6). As seen in Table 2, the temperature sensitive *ho::lacZ* expression phenotype of many of the ANK mutants, including *swi6-406*, could be suppressed by elevated levels of *NHP6A*. The one exception was *swi6-401*, which is the most defective mutant of the set tested. In addition, *NHP6A* could not suppress the *ho::lacZ* expression defect of *swi6-Δ21*, which carries a deletion of the putative leucine zipper in *SWI6*. Thus, *NHP6A* displays allele-specific suppression. It enhances transcription by some temperature-labile Swi6 proteins and has no detectable suppressing activity with others. This could indicate a direct interaction between Nhp6A and Swi6, which is disrupted by only a subset of the Swi6 mutants. However, since Nhp6A suppresses all but the most defective alleles of *SWI6*, this could also be explained if there is a threshold for detection of suppression and some mutants fall below this threshold.

Table 2. *NHP6A* is an allele specific suppressor of *SWI6*

Strain number	Allele	Mutation	ANK repeat affected	Suppression by *NHP6A*
BY1954	*swi6-405*	N330T, N500Y	1 4	+
BY1956	*swi6-406*	T326I, T402S	1 3	+
BY1957	*swi6-402*	A329T, K339R, I395T	1 3	+/-
BY1958	*swi6-401*	K357E, N469I, A494G	2 4	-
BY1959	*swi6-A1*	T326I, I328L L453S, K501R	1 spacer 4	+
BY1960	*swi6-407*	G347N	2	+
BY1693	*swi6-Δ21*	D(E585-L606)	none	-
BY2223	*swi6-4*	G470A, T472A, L474A	4	+

Mutations are described by the original residue, its position, and the substituting residue(for example N330T is glutamine at position 330 in the Swi6 protein changed to threonine). The subdivision of the ANK region into four full copies of the repeats is as in (27). The spacer region is a stretch of amino acids between the third and forth repeats of Swi6 that is not conserved among the members of the Swi4/Swi6 family (27). Plasmid pN-5 bearing *NHP6A* gene was transformed into the strains carrying indicated mutant *SWI6* alleles. These transformants were then grown at 37° (or at 30°, in the case of *swi6Δ-21*) on plates and subjected to X-gal assays. Strains transformed by pRS426 vector served as controls for these assays.

β-galactosidase assays show that suppression by the 2μm plasmid-borne *NHP6A* or *MSN1* of the *ho::lacZ* expression defect in *swi6-405* or *swi6-406* cells at 37° is low but well above background. For example, β-galactosidase activities for *swi6-406* transformed with *MSN1* (pM-2) or *NHP6A* (pN-5) are 22 and 15 units, respectively, compared to 5 units for the vector-transformed control. To see if this increased expression occurred at the transcription level, we analyzed levels of *ho::lacZ* mRNA in these strains by S1 protection. *ho::lacZ* mRNA could not be detected in the *swi6-405* and *swi6-406* strains at 37° (data not shown), and at 25° and 30°, it was noticeably higher in these strains transformed with high copy *NHP6A* and *MSN1* plasmids as compared to the same strains carrying the vector alone (Figure 1b, c, d). A similar result was obtained when *MSN1* plasmid was transformed into *swi6-405* (Figure 1d). These data show that both *MSN1* and *NHP6A* exert their function at the mRNA level, rather than by affecting β–galactosidase stability or activity.

Nhp6A has a close homolog, Nhp6B, which has a set of properties indistinguishable from Nhp6A. The two proteins may have overlapping functions, since only deletion of both genes has a discernible phenotype (28). However, *NHP6B* was not among the suppressors that we isolated. Thus, we tested *NHP6B* directly for suppression of *SWI6* ANK mutations. When expressed from a high copy vector (pDK267), *NHP6B* also suppresses *swi6-405*. It is a weaker suppressor than *NHP6A* (pDK268, Figure 1b), but this difference may be due to the lower levels of *NHP6B* expression, which could also explain why different *NHP6A*-expressing plasmids suppress the *ho::lacZ* mRNA transcription defect to slightly different degrees (Figure 1b).

Swi6-405 and Swi6-406 are maintained at lower levels than the wild type protein, so one indirect mechanism of suppression by *NHP6A* and *B* could be that of increasing expression of *swi6-405*, *swi6-406,* or the *SWI4* gene. To test this possibility, we looked at *SWI4* transcription in the *swi6-405* strains with or without the elevated level of Nhp6A and found no difference in *SWI4* mRNA levels (data not shown). We were not able to test if the Swi4 protein levels were affected, since available antibodies do not detect endogenous levels of this protein. However, since Nhp6A and B are generally considered to be involved in DNA metabolism rather than in protein stability, the fact that *SWI4* transcript levels are not affected by Nhp6A overproduction makes it likely that the protein levels are also unaltered. We also measured the levels of Swi6-405 protein at 37° in cells transformed with vector alone or with *NHP6A*- or *NHP6B*-expressing plasmids, and found that the mutant Swi6 accumulated to the same level in all strains tested (Figure 1a). Thus, the suppression by Nhp6A or B proteins cannot be attributed to the increase in *SWI4* or *SWI6* expression.

***MSN1* and *NHP6A* are involved in *HO* transcription:** To see if *MSN1* and *NHP6A* are normally involved in the transcription of the Swi4/Swi6-regulated promoters, we isolated mRNA from exponentially growing cultures of strains with or without *MSN1*, or *NHP6A* and *B* gene products and compared the levels of *HO* and *CLN1* transcripts in these strains to the wild type strain by S1 protection (Figure 2). The *msn1Δ* strain expresses about three to five-fold less *HO* transcript and the *nhp6abΔ* strain shows a two-fold drop in *HO* transcript compared to wild type. Interestingly, there is little or no effect of *msn1Δ* on

another Swi4/Swi6-regulated promoter, *CLN1* (data not shown) but the *nhp6abΔ* has a similar two-fold effect on *CLN1* (Figure 2c, d).

Both *msn1Δ* and *nhp6abΔ* deletions have complex phenotypes and they affect growth rates. To see if reduced *CLN1* and *HO* levels in these strains could be an indirect effect of a change in cell cycle progression, we used FACS analysis to determine the distribution of cells within the cell cycle in these strains. Both *msn1Δ* and *nhp6abΔ* have an increased proportion of cells in the G1 phase of the cell cycle in comparison with the wild type (Figure 2d). Since the *HO* and *CLN1* genes are expressed predominantly in late G1, the higher proportion of G1 cells in *msn1Δ* and *nhp6abΔ* cultures is likely to mask the actual extent of reduction of *HO* or *CLN1* transcription in these strains rather than be responsible for it.

***NHP6A* and *B* genes can suppress the caffeine sensitivity of the *swi6-405* allele:** The *NHP6A* gene has been implicated as a downstream target of the Slt2/Mpk1 MAP kinase pathway that leads from Pkc1 and is involved in growth control and cell morphogenesis (28), in part because overexpression of *NHP6A* or *B* suppresses several Slt2 pathway defects, including the caffeine sensitivity of *slt2* mutants. Since *swi6Δ* mutants are also sensitive to caffeine (29), we examined whether the *SWI6* ANK mutant, *swi6-405*, is sensitive to caffeine, and if so, whether this defect can be suppressed by overexpression of *NHP6A* or *B*. The *swi6-405* strain cannot grow at 37° on plates containing 4.5 mM caffeine, whereas the wild type cells were capable of growing on these plates. However, when *NHP6A* and *B* genes expressed from high copy plasmids were transformed into the *swi6-405* strain, they restored the ability of these cells to grow on plates containing up to 5.5 mM caffeine.

Nhp6A probably affects Swi4/Swi6 DNA binding indirectly: To see whether there is a direct interaction between Nhp6A and Swi6, we immunoprecipitated Swi4 or Swi6 proteins out of wild type extracts carrying HA-Nhp6A and then immunoblotted with anti HA antibodies to detect HA-Nhp6A. Despite the fact that this HA-tagged Nhp6A is functional and can suppress the *swi6-405* transcription defect (Figure 1b), there was no indication that HA-Nhp6A coprecipitates either with Swi4 or Swi6 under the same conditions that we use to detect Swi4/Swi6 association (6).

We then prepared fusions of Nhp6A and Msn1 proteins with Gst. The *GST-NHP6A* and *GST-MSN1* fusions were put under the control of *GAL1-10* promoter and expressed in a wild type strain. Because of their toxicity when overproduced, we purified these Gst fusions from cells grown in raffinose and then induced by galactose addition for only 3-4 hours. Though we could purify the fusion proteins from these cells under low stringency conditions, there was no detectable Swi6 copurifying with either of them. We also purified these Gst fusion proteins from *E. coli* on glutathione beads and then incubated the fusion-bound beads with recombinant Swi6 (6), or in vitro translated Swi4/Swi6 complex (21). Even under these conditions, we could not detect interaction between Swi4, or Swi6, and the fusion proteins.

Nhp6A may not directly associate with Swi6, yet it could facilitate the binding of the Swi4/Swi6 complex to SCBs by inducing a favourable bend in DNA. Thus, we tested the involvement of Nhp6A in the Swi6 complex formation on DNA. Band shift analysis

was carried out with Swi4/Swi6 complexes obtained by in vitro translation of Swi4 in the presence of recombinant Swi6. These complexes appeared to be identical to the complex observed in whole cell extracts, in that they migrated with the same mobility, contained both Swi4 and Swi6 proteins, as judged by its supershifting by Swi4 and Swi6 antibodies, and were specific to SCB elements of the *HO* promoter fragment (Figure 3a and data not shown). The purified Gst-Nhp6A fusion was first tested in gel retardation assays with the *HO* promoter fragment. The Gst-Nhp6A fusion, but not Gst alone, was able to nonspecifically bind DNA (Figure 3b, lane 8). We also cleaved the Gst moiety off the Gst-Nhp6A fusion with thrombin and tested the released Nhp6A in gel retardation assays. As anticipated, thrombin cleavage of Gst-Nhp6A released a DNA binding component that forms a much smaller complex on DNA (Figure 3b, lane 15). However, both Gst-Nhp6A and Nhp6A were able to bind DNA and both could form a series of bandshifts indicating that multiple Nhp6A molecules bound simultaneously to one DNA molecule. Next, the in vitro translated Swi4/Swi6 complex was mixed together with varying amounts of Gst-Nhp6A or Nhp6A and was added to the *HO* promoter fragment (Figure 3b, lanes 5-7 and 12-14). These reactions were compared to the ones in which Swi4/Swi6 complex was mixed with Gst only (lanes 1-3), thrombin cleavage mixture only (lanes 9-11), and Gst-Nhp6A or Nhp6A mixed with the rabbit reticulocyte lysate (lanes 8 and 15). The amount of the DNA-bound Swi4/Swi6 complex was unaffected by the addition of Gst-Nhp6A (compare lanes 1-3 with 5-7) and slightly reduced upon the addition of Nhp6A (compare lanes 9-11 and 12-14). These results indicate that there is no cooperation between Nhp6A and Swi4/Swi6 in binding to DNA under the conditions of this assay. It is also worth noting that there appeared to be a negative effect on the amount of DNA-bound Gst-Nhp6A and Nhp6A if the Swi4/Swi6 complex was present in the reaction (compare lanes 7 to 8 and 14 to 15). Since the *HO* DNA was in excess in the reaction, this cannot be attributed simply to competition for binding sites.

Conclusions

ANK repeats were first found in the Swi6 transcription factor of *Saccharomyces cerevisiae* and since then were identified in many proteins of eukaryotes and prokaryotes. These repeats are thought to serve as protein association domains. In Swi6, ANK repeats affect DNA binding of both the Swi4/Swi6 and Mbp1/Swi6 complexes. We have previously described generation of random mutations within the ANK repeats of Swi6 that render the protein temperature sensitive in its ability to activate *HO* transcription. Two of these *SWI6* mutants were used in a screen for high copy suppressors of this phenotype. We found that *MSN1*, which encodes a transcriptional activator, and *NHP6A*, which encodes an HMG-like protein, are able to suppress defective Swi6 function. Both of these gene products are involved in *HO* transcription, and Nhp6A may also be involved in *CLN1* transcription. Moreover, since overexpression of *NHP6A* can suppress caffeine sensitivity of one of the *SWI6* ANK mutants, *swi6-405*, other *SWI6*-dependent genes may also be affected by Nhp6A. We hypothesize that Nhp6A and Msn1 modulate Swi6-dependent gene transcription indirectly, through effects on chromatin structure or other transcription factors, since we have not been able to demonstrate that either Msn1 or Nhp6A interact with the Swi4/Swi6 complex.

Recommendations pertaining to statement of work

This suppressor screen was carried out with two mutants which were largely uncharacterized when the study was done. Subsequent modelling studies have allowed us to define mutations on the surface of Swi6 that are defective for Swi6 function. These are much more likely to define surfaces critical for protein-protein interaction, and thus are a much better starting point for the suppressor screens that were proposed originally in Task5 of the Statement of Work. As such, we have extended the duration of this study for an additional year, with the goal of identifying critical surface residues in Swi6 and repeating the suppressor screen.

References

1. Hussussian, C. J., Struewing, J. P., Goldstein, A. M., Higgins, P. A. T., Ally, D. S., Sheahan, M. D., Clark, W. H., Tucker, M. A. & Dracopoli, N. C. (1994) *Nature Genet* **8**, 15-21.

2. Tevelev, A., Byeon, I. L., Selby, T., Ericson, K., Kim, H., Kraynov, V. & Tsai, M. (1996) *Biochem.* **35**, 9475-9487.

3. Gorina, S. & Pavletich, N. P. (1996) *Science* **274**, 1001-1005.

4. Breeden, L. & Nasmyth, K. (1987) *Nature* **329**, 651-654.

5. Lux, S. E., John, K. M. & Bennett, V. (1990) *Nature* **344**, 36-42.

6. Sidorova, J. & Breeden, L. (1993) *Mol. Cell. Biol.* **13**, 1069-1077.

7. Andrews, B. J. & Moore, L. A. (1992) *Proc. Natl. Acad. Sci. USA* **89**, 11852-11856.

8. Koch, C., Moll, T., Neuberg, M., Ahorn, H. & Nasmyth, K. (1993) *Science* **261**, 1551-1557.

9. Lowndes, N. F., Johnson, A. L., Breeden, L. & Johnston, L. H. (1992) *Nature* **357**, 505-508.

10. Ogas, J., Andrews, B. J. & Herskowitz, I. (1991) *Cell* **66**, 1015-1026.

11. Dirick, L., Moll, T., Auer, H. & Nasmyth, K. (1992) *Nature* **357**, 508-513.

12. McIntosh, E. M., Atkinson, T., Storms, R. K. & Smith, M. (1991) *Mol. Cell. Biol.* **11**, 329-337.

13. Primig, M., Sockanathan, S., Auer, H. & Nasmyth, K. (1992) *Nature* **358**, 593-597.

14. Andrews, B. J. & Herskowitz, I. (1989) *Nature* **342**, 830-833.

15. Andrews, B. J. & Moore, L. (1992) *Biochem. & Cell Biol.* **70**, 1073-80

16. Partridge, J. F., Mikesell, G. E. & Breeden, L. L. (1997) *J. Biol. Chem.* **272**, 9071-9077.

17. Bork, P. (1993) *Proteins: Structure, Function, and Genetics* **17**, 363-374.

18. Breeden, L. & Mikesell, G. (1991) *Genes & Dev.* **5**, 1183-1190.

19. Heichman, K. A. & Roberts, J. M. (1996) *Cell* **85**, 39-48.

20. Sidorova, J., Mikesell, G. & Breeden, L. (1995) *Mol. Biol. Cell* **6**, 1641-1658.

21. Ewaskow, S. P., Sidorova, J. M., Hendle, J., Emery, J. C., Lycan, D. E., Zhang, K. Y. J. & Breeden, L. L. (1998) *Biochem.* **37**, 4437-4450.

22. Paull, T. T. & Johnson, R. C. (1995) *J. Biol. Chem.* **270**, 8744-8754.

23. Carlson, M. & Botstein, G. (1982) *Cell* **28**, 145-154.

24. Breeden, L. & Nasmyth, K. (1985) *Cold Spring Harbor Symp. Quant. Biol.* **50**, 643-650.

25. Estruch, F. & Carlson, M. (1990) *Nucl. Acids Res.* **18**, 6959-6964.

26. Kolodrubetz, D. & Burgum, A. (1990) *J. Biol. Chem.* **265**, 3234-3239.

27. Breeden, L. (1996) in *Start-specific transcription in yeast.* ed. Farnham, P. J. 95-127.

28. Costigan, C., Kolodrubetz, D. & Snyder, M. (1994) *Mol. Cell. Biol.* **14**, 2391-2403.

29. Igual, J. C., Johnson, A. L. & Johnston, L. H. (1996) *EMBO J.* **15**, 5001-5013.

APPENDICES

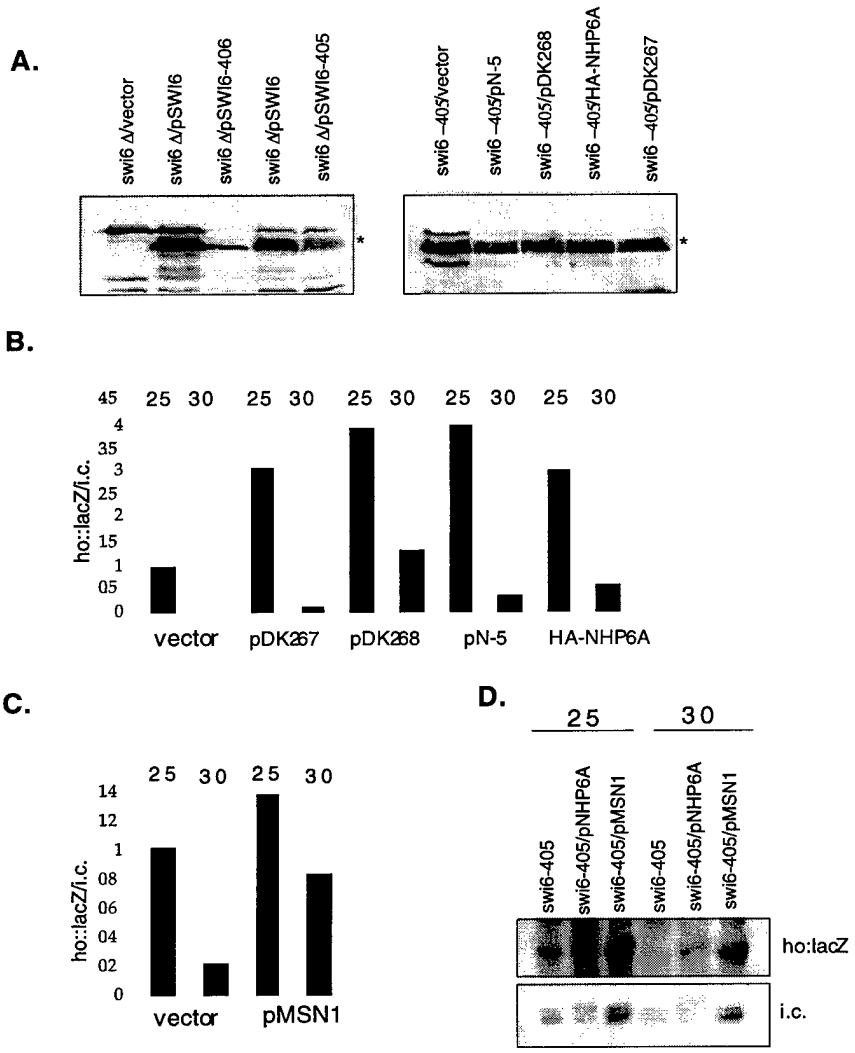

Figure 1. *NHP6A* and *MSN1* suppress *swi6-405* and *swi6-406* ANK mutant *ho::lacZ* transcription defects. **A.** Western blots of Swi6 expressed in the following strains at 37°. BY600 was transformed with pRS316 (vector), pSWI6 pBD1378, pSWI6-405 pBD2031 and pSWI6-406 pBD2046. BY1954 *swi6-405 ho::lacZ* strain was transformed with the vector, pRS426, or *NHP6A* or *B* carrying plasmids pN-5, pDK268, HA-NHP6A, pDK267. Asterisk marks the position of the Swi6 protein. **B.** S1 protection was performed on RNAs isolated from the same set of BY1954 *swi6-405* strains grown at 25° or 30° for 10 hours. Levels of *ho::lacZ* mRNA obtained from 2-3 measurements were quantitated, normalized to the internal control levels (*SIR3* mRNA) and plotted. **C.** S1 protection was performed on RNAs isolated from the BY1956 *swi6-406* strain transformed with vector pRS426 or *MSN1*-carrying pM-2, and grown for 10 hours at 25° or 30°. *ho::lacZ* levels were measured and quantitated as in **B. D.** S1 protection was performed on BY1954 *swi6-405* strain transformed with vector pRS426, pN-5, or c12 (an *MSN1*-carrying clone) and grown at 25° or 30°. Positions of *ho::lacZ* and internal control (*SIR3*) transcripts are marked.

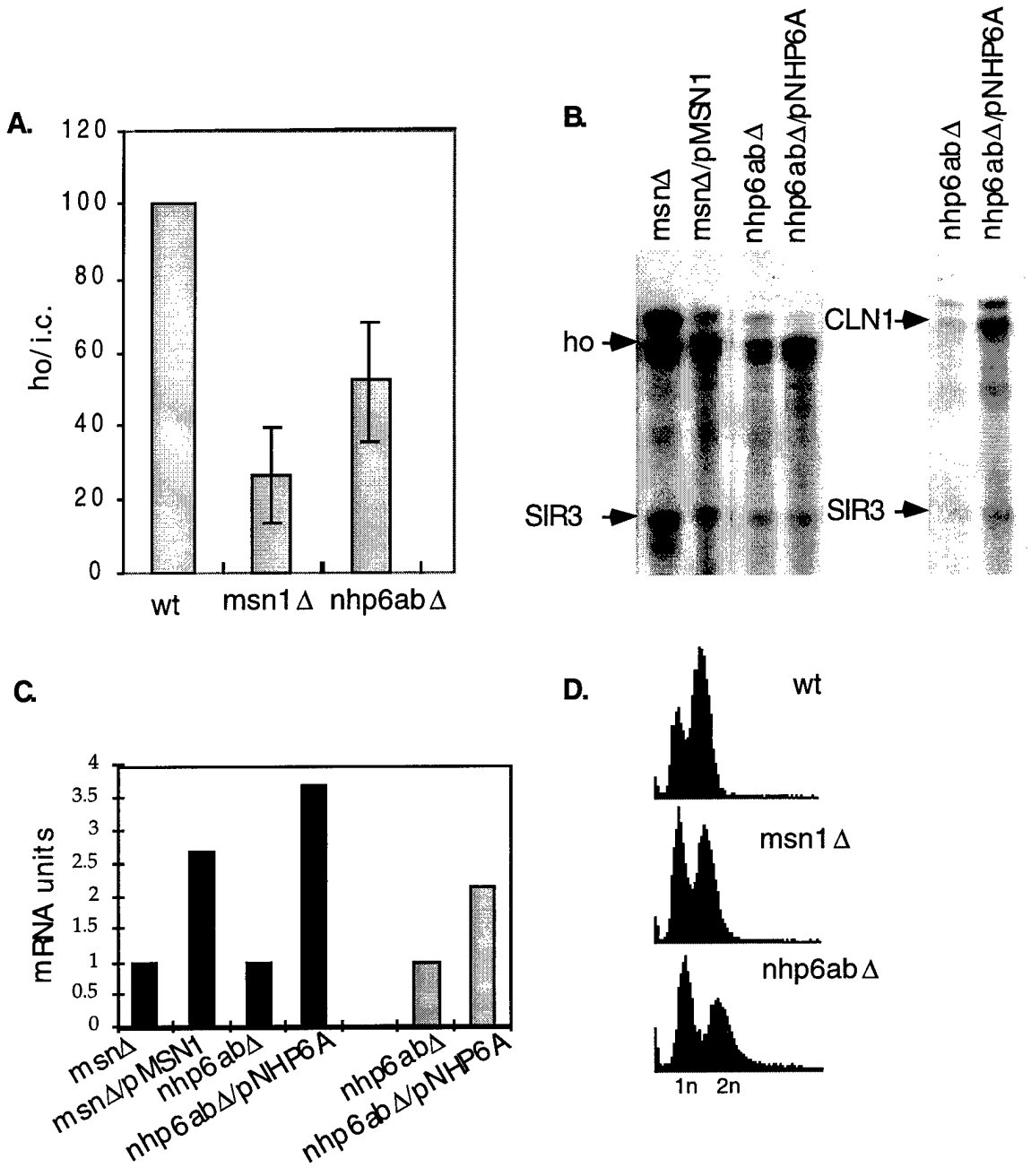

Figure 2. *HO* expression is reduced in *msn1Δ* and *nhp6abΔ* strains. **A.** Five or six S1 protection measurements of the steady state levels of *HO* mRNA were made, quantitated, and normalized to the internal control message (*SIR3* RNA). In each independent experiment, the wild type W303-1a strain measurement was equalled to 100% and the measurements obtained for *msn1Δ* and *nhp6abΔ* strains were calculated as the percentage of the wild type, and then averaged. Average deviation of these measurements is shown by the error bars. **B.** The *msn1Δ* strain was transformed with vector pRS426 (lane 1) or high copy *MSN1* (pM-1) (lane 2), and *nhp6abΔ* was transformed with pBD2076 (a pSH144 Guarente vector version with a *LEU2* marker, {944} lane 3) or high copy *NHP6A* (pN-3) (lane 4). The levels of *HO* or *CLN1* in the resulting strains were measured by S1 protection. *SIR3* mRNA serves as internal control. **C.** Levels of *HO* or *CLN1* were normalized to the levels of the internal control and plotted. Black bars represent *HO* message levels and grey bars correspond to *CLN1* message levels. **D.** Aliquots of cultures used for RNA measurements were fixed, stained with propidium iodide and subjected to FACS analysis.

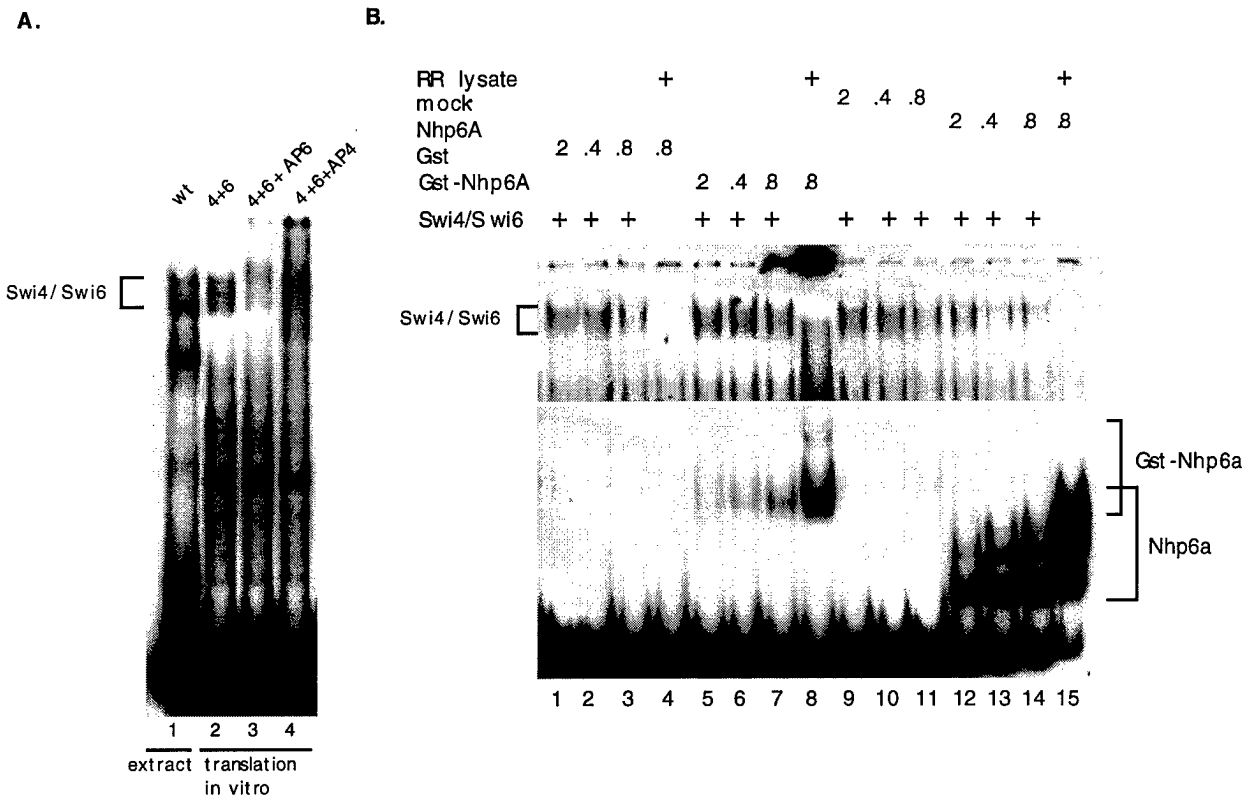

Figure 3. Nhp6A does not cooperate with the Swi4/Swi6 complex in binding to the *HO* promoter. **A.** An *in vitro* translated Swi4/Swi6 complex was mixed with the labelled *HO* promoter DNA, and gel retardation analysis was performed. Lanes 2-4 show the in vitro generated Swi4/Swi6 complex bound to the DNA (translation in vitro). In lanes 3 and 4, 2 and 5 µl of affinity purified Swi6 (AP6) and Swi4 (AP4) antibodies, respectively, were added to the DNA binding reactions. Lane 1 shows the Swi4/Swi6 complex generated on the *HO* promoter by wild type yeast extract. **B.** A gel retardation experiment performed with mixtures of purified Nhp6A and the *in vitro* produced Swi4/Swi6 complex. Two exposures of one gel are shown together to follow both the abundant Nhp6A complexes and the less abundant Swi4/Swi6 complex. The reagents added are listed on the left. Plus indicates additions of either the rabbit reticulocyte lysate-translated Swi4 and recombinant Swi6 (Swi4/Swi6), or the unprogrammed lysate (RR lysate). For this experiment Gst-Nhp6A and Gst were purified from *E. coli* as indicated in Materials and Methods. Nhp6A or a mock preparation of it were obtained by thrombin cleavage of Gst-Nhp6A or Gst immobilized on glutathione beads (see Materials and Methods). Numbers designate the microliter amounts of purified Gst-Nhp6A, Gst, Nhp6A or mock preparation added to the reactions. These proteins were added to the DNA binding reactions together with reticulocyte lysate mixtures and incubated as described in Materials and Methods . Brackets on the right show the positions of the DNA-bound Nhp6A and Gst-Nhp6A complexes and the arrow on the left points at the position of the Swi4/Swi6 complex.